

(12) PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. AU 199955426 B2
(10) Patent No. 758019

(54) Title
Anthrax vaccine

(51)⁶ International Patent Classification(s)
C12N 015/31

(21) Application No: 199955426 (22) Application Date: 1999 . 07 . 09

(87) WIPO No: WO00/02522

(30) Priority Data

(31) Number (32) Date (33) Country
60/092416 1998 . 07 . 10 US

(43) Publication Date : 2000 . 02 . 01

(43) Publication Journal Date : 2000 . 04 . 13

(44) Accepted Journal Date : 2003 . 03 . 13

(71) Applicant(s)
U.S. Medical Research Institute of Infectious Diseases

(72) Inventor(s)
John S. Lee; Peter Pushko; Michael D Parker; Jonathan F
Smith; Susan L. Welkos

(74) Agent/Attorney
FISHER ADAMS KELLY.GPO Box 1413,BRISBANE QLD 4001

(56) Related Art
WO 98/08952
IACONO-CONNORS LC ET AL INFEC AND IMM 59(6):1961-1965
IVINS BE ET AL, INFE AND IMMUN 54(2): 531-542

pages 1/5-5/5, drawings, replaced by new pages 1/5-5/5; pages 1-4, sequence listing, replaced by new pages 1-8; due to late transmittal by the receiving Office

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

IP AUSTRALIA

18 APR 2001

**(19) World Intellectual Property Organization
International Bureau**



Abstract

(43) International Publication Date
20 January 2000 (20.01.2000)

PCT

(10) International Publication Number
WO 00/02522 A3

(51) International Patent Classification⁷: C12N 15/31,
C07K 14/32, A61K 39/07, C12N 7/01, 1/21

6936 Eylers Valley Flint Road, Sabillasville, MD 21780 (US). WELKOS, Susan, L. [US/US]; 1594 Dockside Drive, Frederick, MD 21701 (US).

(21) International Application Number: PCT/US99/15568

(22) International Filing Date: 9 July 1999 (09.07.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 60/092,416 10 July 1998 (10.07.1998) US

(71) Applicant (for all designated States except US): U.S. MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES [US/US]; Department of the Army, 504 Scott Street, Frederick, MD 21702 (US).

(72) Inventors; and

(75) **Inventors/Applicants (for US only):** LEE, John, S. [US/US]; 1807 Elizabeth Court, Hagerstown, MD 21740 (US). PUSHKO, Peter [US/US]; 917 Seminole Road, Frederick, MD 21701 (US). PARKER, Michael, D. [US/US]; Apartment T-13, 1001 Carroll Parkway, Frederick, MD 21701 (US). SMITH, Jonathan, F. [US/US];

(74) Agents: HARRIS, Charles, H.; United States Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD 21702 et al. (US).

(81) Designated States (national): AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW.

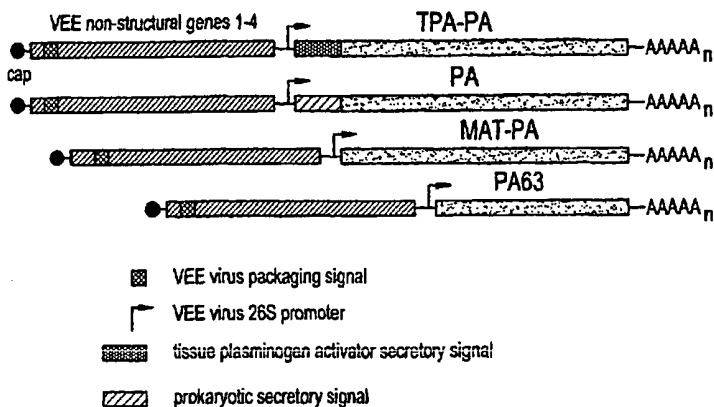
(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— With international search report.

[Continued on next page]

(54) Title: ANTHRAX VACCINE



(57) **Abstract:** Using the nontoxic protective antigen PA protein from *B. anthracis*, a method and composition for use in inducing an immune response which is protective against anthrax in subjects is described.

TITLE OF THE INVENTIONAnthrax Vaccine

5

10

FIELD OF THE INVENTION

This invention relates to vaccines for bacterial toxins from *Bacillus anthracis*.

15

INTRODUCTION

Anthrax is a disease caused by the sporulating bacteria *Bacillus anthracis*. Humans working with animal products are at risk from contracting anthrax. Areas such as Iran, Turkey, Iraq, Pakistan, and sub-Saharan Africa are hyperendemic for anthrax, although the organism can be found in most areas of the world. Anthrax manifests disease in three different ways. Inhalation, gastrointestinal, and cutaneous anthrax result from inhaling spores, ingesting spores in contaminated meat, or contacting spores in an open wound, respectively. Untreated inhalation or gastrointestinal anthrax has a case fatality rate of essentially 100 percent while cutaneous anthrax has a case fatality rate of up to 25 percent. Previous research has shown that the protective antigen (PA) produced by *B. anthracis* can protect mice from anthrax. Even though the Anthrax vaccine is FDA licensed, reactogenicity is mild to moderate.

Therefore, there is a need for an efficacious vaccine for anthrax useful for protecting humans.

SUMMARY OF THE INVENTION

5 The present invention satisfies the need discussed above. The present invention relates to a method and composition for use in inducing an immune response which is protective against infection with anthrax.

10 In this vaccine strategy, a gene coding for a protein of interest is cloned into a VEE virus vector in place of the VEE virus structural genes; the result is a self-replicating RNA molecule, a replicon, that encodes its own replicase and transcriptase functions,
15 and in addition makes abundant quantities of mRNA encoding the foreign protein. When replicon RNA is transfected into eukaryotic cells along with two helper RNAs that express the VEE structural proteins (glycoproteins and nucleocapsid), the replicon RNA is
20 packaged into VEE virus-like particles by the VEE virus structural proteins, which are provided in trans. Since the helper RNAs lack packaging signals necessary for further propagation, the resulting VEE replicon particles (VRPs) which are produced are
25 infectious for one cycle but are defective thereafter. Upon infection of an individual cell with a VRP, an abortive infection occurs in which the infected cell produces the protein of interest in abundance, is ultimately killed by the infection, but does not
30 produce any viral progeny (Pushko et al., 1997, *Virology* 239, 389-401).

 The PA gene which contains a prokaryotic secretory signal and the entire 83 kDa coding sequence (Welkos et al., 1988, *Gene* 69, 287-300), was inserted

into the VEE replicon vaccine vector (Figure 1) and have demonstrated high level expression of this bacterial protein in eukaryotic cells in culture. Mice, either the C57BL/6 strain or the A/J strain, inoculated with VRP containing the PA-replicon produced high specific antibody titers and were protected from developing anthrax when challenged subcutaneously with *B. anthracis*.

Therefore, it is one object of the present invention to provide a VEE virus replicon vector comprising a VEE virus replicon and DNA fragments encoding the PA protein from *B. anthracis*.

It is another object of the present invention to provide a self replicating RNA comprising the VEE virus replicon and any of the *B. anthracis* fragments described above.

It is another object of the present invention to provide infectious VEE virus replicon particles produced from the VEE virus replicon RNA described above.

It is further an object of the invention to provide an immunological composition for the protection of mammals against *B. anthracis* infection comprising VEE virus replicon particles containing any of the *B. anthracis* fragments described above or a combination of different VEE virus replicons each having a different *B. anthracis* fragment.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. Diagram of the protective antigen (PA) replicon constructs. The PA replicons are similar to

the full-length VEE RNA except that the open reading frame encoding the VEE structural proteins was replaced with the PA genes.

Figure 2. Western blot of BHK cell lysates showing expression of anthrax protective antigen from recombinant VEE replicons. a) uninfected cell lysate; b) recombinant expression product from *B. anthracis*; c) infected cell lysate.

Figure 3A and 3B. ELISA titers for mice (A, C57Bl/6 mice; B, A/J mice) immunized with PA-VRP. GMTs only includes those mice that survived challenge. a) Received 0.2 ml on day 0 and 28. b) Received 10^7 iu on day 0 and 28. c) Received 10^7 iu PA-VRP on day 0, 28, 56, and 84; C57Bl/6 mice that died, titer = 100; A/J mice that died, titer = 400. d) Received 10^7 iu PA-VRP on day 0, 28, and 56; C57Bl/6 mice that died, titers = 400, 100, 100; A/J mice that died, titers = 102400, 100, 1600. e) Received 10^7 iu PA-VRP on day 0, and 28; C57Bl/6 mice that died, GMT = 1313; A/J mice that died, GMT = 1766. Mice were challenged 28 days after last inoculation with 18-32 LD₅₀ Sterne strain for C57Bl/6 and 12-25 LD₅₀ Sterne strain for A/J mice.

Figure 4. Schematic representation of constructs containing PA.

DETAILED DESCRIPTION

In the description that follows, a number of terms used in recombinant DNA, virology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Replicon. A replicon is equivalent to a full length virus from which all of the viral structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site. Transcription of the RNA from the replicon yields an RNA capable of initiating infection of the cell identically to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA *in trans*. This is typically done with two helpers also called defective helper RNAs. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for subgenomic mRNA transcription and the sequences for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro* and co-transfected with replicon RNA. Because the replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the cell. The particles can then be inoculated into animals similar to parent virus. The replicon

particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only the viral nonstructural proteins and the product of the
5 heterologous gene cloned in place to the structural proteins.

The VEE virus replicon is a genetically reorganized version of the VEE virus genome in which the structural proteins genes are replaced with a gene
10 from an immunogen of interest, in this invention, the *B. anthracis* proteins. The result is a self replicating RNA (replicon) that can be packaged into infectious particles using defective helper RNAs that encode the glycoprotein and capsid proteins of the VEE
15 virus.

Subject. Includes both human, animal, e.g., horse, cattle, donkey, monkey, pig, dog, guinea pig, mouse, hamster, avian e.g., chicken, pheasant or
turkey, fish and other marine animals, and insects
20 such as mosquito.

In one embodiment, the present invention relates to a recombinant DNA molecule that includes a VEE replicon and a DNA sequence encoding the non-toxic
25 protective antigen (PA) from *B. anthracis*. The sequence for PA has been determined and has been deposited in GenBank at accession no. M22589. The PA sequence encodes a prokaryotic secretory signal in addition to the entire sequence encoding the 83 Kd PA
30 (SEQ ID NO:1). Other nucleic acid sequences related to this invention include sequences wherein the secretory signal has been removed (MAT-PA) (SEQ ID NO:2), or replaced with other secretory signals known to people in the art such as the tissue plasminogen
35 activator (TPA) secretory signal resulting in a DNA

fragment encoding TPA-PA (SEQ ID NO:3). Nucleic acid sequences included in this invention are sequences encoding the active form of PA, a 63 kDa protein, termed PA63 (SEQ ID NO:4). In addition, isolated
5 nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the *B. anthracis* proteins described and specified in
10 SEQ ID NO:5 (TPA-PA), SEQ ID NO:6 (PA), SEQ ID NO:7 (MAT-PA), and SEQ ID NO:8 (PA63). Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate
15 variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E.coli*).

Nucleic acid molecules of the present invention
20 may be in the form of RNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the
25 sense strand, or it may be the non-coding strand, also referred to as the antisense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For
30 example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or
35 substantially) DNA molecules in solution. Isolated

RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such
5 molecules produced synthetically.

The present invention is further directed to nucleic acid molecules encoding portions or fragments of the nucleotide sequences described herein. Fragments include portions of the nucleotide sequences
10 of at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence is
15 position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotide bases in length or any integer between 10 and the length of an entire nucleotide sequence minus 1.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the *B. anthracis* polypeptides described above. Variants may occur naturally, such as a
25 natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus of a chromosome of an organism. Non-naturally occurring variants may be produced by known mutagenesis techniques. Such
30 variants include those produced by nucleotide substitution, deletion, or addition of one or more nucleotides in the coding or noncoding regions or both. Alterations in the coding regions may produce conservative or nonconservative amino acid
35 substitutions, deletions, or additions. Especially

preferred among these are silent substitutions, additions, and deletions which do not alter the properties and activities of the *B. anthracis* polypeptides disclosed herein or portions thereof.

- 5 Also preferred in this regard are conservative substitutions.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The
10 vector can take the form of a plasmid, phage, cosmid, YAC, eukaryotic expression vector such as a DNA vector, *Pichia pastoris*, or a virus vector such as for example, baculovirus vectors, retroviral vectors or adenoviral vectors, and others known in the art. The
15 cloned gene may optionally be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences, or sequences which may be inducible and/or cell type-specific. Suitable promoters will be known to a person with
20 ordinary skill in the art. The expression construct will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. When the DNA sequences described above are in a
25 replicon expression system, such as the VEE replicon described above, the proteins can be expressed *in vivo*. The DNA sequence for any of the *B. anthracis* proteins described above can be cloned into the multiple cloning site of a replicon such that
30 transcription of the RNA from the replicon yields an infectious RNA containing the *B. anthracis* protein or proteins of interest. Use of helper RNA containing sequences necessary for encapsulation of the viral transcript will result in the production of viral
35 particles containing replicon RNA which are able to

infect a host and initiate a single round of replication resulting in the expression of the *B. anthracis* proteins. Such replicon constructs include those listed in Table 1.

5	Table 1		
	Replicon		
	<u>Plasmid Name</u>	<u>expresses</u>	
	p3014-TPA-PA	TPA signal, 5 amino acids of C terminal end of prokaryotic secretory signal, and the 83kDa mature protein.	10
	p3014-PA	29 amino acid prokaryotic secretory signal, and the 83kDa mature protein secreted by <i>B. anthracis</i>	15
	p3014-MAT-PA	83kDa mature protein	
	p3014-PA63	63kDa protein (minus 20kDa N-terminal end that is naturally cleaved off by furin proteases at host cell membrane surface)	20
	p3014-sub-domains	subdomain #1: from nucleotide 1891 to 2391; subdomain #2: from nucleotide 2392 to 2658; subdomain #3: from nucleotide 2659 to 3351; subdomain #4: from nucleotide 3352 to 3669; subdomain #5: from nucleotide 3670 to 4098 (Subdomains are numbered according to Welkos et al., 1988, supra, determined on subdomains present in the crystal structure, Petosa, et al., 1997, Nature 385, 833-838).	25
			30
			35

The VEE constructs containing anthrax proteins can be used as a DNA vaccine, or for the production of RNA molecules as described below.

40 In another embodiment, the present invention relates to RNA molecules resulting from the transcription of the constructs described above. The RNA molecules can be prepared by *in vitro* transcription using methods known in the art and
 45 described in the Examples below. Alternatively, the

RNA molecules can be produced by transcription of the constructs *in vivo*, and isolating the RNA. These and other methods for obtaining RNA transcripts of the constructs are known in the art. Please see Current
5 Protocols in Molecular Biology. Frederick M. Ausubel et al. (eds.), John Wiley and Sons, Inc. The RNA molecules can be used, for example, as a nucleic acid vaccine, or to transfect cells along with RNA from helper plasmids, one of which expresses VEE
10 glycoproteins and the other VEE capsid proteins, as described above, in order to obtain replicon particles.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection,
15 electroporation, infection, and other methods known in the art and described in standard laboratory manuals such as Current Protocols in Molecular Biology, Ausubel, F. M. et al. (Eds), Wiley & Sons, Inc. All documents cited herein *supra* and *infra* are hereby
20 incorporated in their entirety by reference thereto.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for
25 example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to rat and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences
30 when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions,

and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fritsch and Sambrook, Molecular Cloning: A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of protein of the invention, such as glutathione S-transferase. The recombinant molecule can be suitable for transfecting eukaryotic cells, for example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as baby hamster kidney (BHK) cells, MRC-5 cells and vero cells, to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase

expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. The transformed or transfected host cells can be used as a source of
5 DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein or polypeptide specified as amino acid sequence in SEQ ID NO:5 (TPA-PA), SEQ ID
10 NO:6 (PA), SEQ ID NO:7 (MAT-PA), and SEQ ID NO:8 (PA63).

A polypeptide or amino acid sequence derived from the amino acid sequences mentioned above, refers to a polypeptide having an amino acid sequence identical to
15 that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically
20 identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including
25 for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, such as adjuvants for example.

30 The recombinant or fusion protein can be used as a vaccine for immunity against anthrax infection or as a diagnostic tool for detection of bacillus anthracis. The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit
35 anthrax or *B. anthracis* proteins, such as host

proteins or chemically derived agents or other proteins which may interact with *B. anthracis* proteins of the present invention to inhibit its function. A method for testing the effectiveness of an anti-anthrax drug or agent can for example be the rat anthrax toxin assay (Ivins et al. 1984, *Infec. Immun.* 52, 454-458 and Ivins et al. 1986) or a skin test in rabbits for assaying antiserum against anthrax toxin (Belton and Henderson, 1956, *Br. J. Exp. Path.* 37, 156-160).

In another embodiment, the present invention relates to an anthrax vaccine comprising one or more replicon particles derived from one or more replicons encoding one or more *B. anthracis* proteins or polypeptides as described above. The present invention relates a method for providing immunity against anthrax said method comprising administering one or more replicon particles containing any combination of the *B. anthracis* proteins to a subject such that a protective immune reaction is generated.

Vaccine formulations of the present invention comprise an immunogenic amount of a replicon particle, resulting from one of the replicon constructs described above, or a combination of replicon particles as a multivalent vaccine, in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered. An amount of from about 10^2 to 10^7 per dose is suitable, more or less can be used depending upon the age and species of the subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the replicon particles disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, orally and by topical application of the virus (typically carried in the pharmaceutical formulation) to an airway surface. Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the replicon as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered.

When the replicon RNA or DNA is used as a vaccine, the replicon RNA or DNA can be administered directly using techniques such as delivery on gold beads (gene gun), delivery by liposomes, or direct injection, among other methods known to people in the art. Any one or more constructs or replicating RNA described above can be use in any combination effective to elicit an immunogenic response in a subject. Generally, the nucleic acid vaccine administered may be in an amount of about 1-5 ug of

nucleic acid per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

The following MATERIALS AND METHODS were used in the examples that follow.

Plasmids. Construction of the VEE replicon, capsid (C-) helper, and glycoprotein (GP-) helper plasmids was previously described (Pushko, 1997, supra). The PA gene from nucleotide 1804 to 4098 (SEQ ID NO:1) (Welkos et al., 1988, supra) was cloned into the VEE replicon plasmid as a BamHI/BamHI fragment utilizing a shuttle vector. TPA-PA (SEQ ID NO:3), MAT-PA (SEQ ID NO:2), and PA63 (SEQ ID NO:4) were PCR amplified using forward and reverse primers which also contained the ClaI recognition site. The PCR products were agarose gel purified and ligated into the T/A vector pCR2.1 (Invitrogen, Inc.). The PA genes, after

digestion of pCR2.1-PA's with ClaI, were ligated into the VEE replicon plasmid. The Lassa nucleocapsid replicon (Lassa N-replicon) was constructed as previously described (Pushko, 1997, supra) and used as a negative control replicon.

Production of VRP. Plasmid templates for the PA-replicons, C-helper, GP-helper, and the Lassa N-replicon were linearized by digestion with NotI at a unique site downstream from the coding sequences, and capped run-off transcripts were prepared *in vitro* using T7 RNA polymerase. Packaging of the replicons into VEE replicon particles (VRPs) was accomplished by electroporating the replicon RNA and the two helper RNAs into BHK cells. VRPs were harvested between 20 and 27 hours after transfection and purified from cell culture supernatants by ultracentrifugation through a discontinuous sucrose gradient (20%). After reconstituting the pelleted VRP in 1/50 volume phosphate buffered saline, the VRPs were stored at -70°C.

Analysis of expression products and titration of VRP. Subconfluent monolayers were infected with PA-VRP's (either TPA-PA, PA, MAT-PA, or PA63) or Lassa N-VRP (m.o.i. = 2) or cell suspensions were electroporated with replicon RNA. Cells were harvested at approximately 20-24 hours and expressed proteins were separated by SDS-PAGE. Visualization of PA proteins was accomplished using a chemiluminescence western blot assay and antibodies specific for each protein. Titration of VRPs was accomplished by infecting subconfluent monolayers with increasing dilutions of purified VRP. Antigen positive cells were visualized in an indirect immunofluorescence assay using a monoclonal antibody specific for each protein,

or in a direct immunofluorescence assay using an FITC-conjugated monkey anti-Lassa serum.

Immunization of mice. Mice were inoculated 1 to 4 times at 28 day intervals with 10^5 to 10^7 infectious units (iu) of either PA-VRP or Lassa N-VRP (negative control). Positive control mice for the anthrax study were inoculated subcutaneously with 0.2 ml of anthrax vaccine at 28 day intervals. Serum for ELISA was obtained 2 days before each inoculation and 3 days before challenge.

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with protective antigen (gift from Joe Farchaus, Bacteriology Division) (1 ug/ml) in PBS and allowed to absorb overnight at 4°C. Four fold serum dilutions in blocking buffer were applied to the plates and incubated at 37°C for 1 hour. After washing, an anti-mouse secondary antibody (HRP conjugated) was added to the plate and incubated for an additional hour at 37°C. After washing, bound antibody was detected colorimetrically using ABTS as a substrate.

Challenge of mice.

Anthrax challenge: C57BL/6 or A/J mice were challenged subcutaneously with 12-32 LD₅₀ *B. anthracis* (Sterne) 31 days after the last inoculation.

Example 1

Packaging and expression of PA-replicon

The PA-replicon was efficiently packaged into VRPs using the double helper system. Stock solutions contained about 10^8 infectious units of purified PA-VRP per milliliter. No replication competent VEE virus was detected in any of the preparations. A western blot of cell lysates, generated from BHK cells infected with PA-VRP, demonstrates a product of

approximately 85 kDa (Figure 2). Because the PA-VRP expression product contains a 29 amino acid prokaryotic secretory signal, which is cleaved from the standard, the PA product from the BHK cells appears larger than the PA standard.

Example 2

Protection against challenge with *B. anthracis*

The results of the animal studies demonstrated that PA from *B. anthracis* could be used to immunize and protect mice from a lethal challenge of *B. anthracis* (Sterne strain). Although the Sterne strain is attenuated in some animals, it is virulent in mice. Controls in the experiment included mice vaccinated with the current human vaccine (anthrax vaccine absorbed, AVA) which protected all mice, and mice immunized with the Lassa N-replicon which expressed an antigen irrelevant to anthrax protection which failed to protect the mice. Figure 3A shows ELISA titers and survival for C57Bl/6 mice inoculated with two to four doses of PA-VRP. Seventy percent of the C57Bl/6 mice which received three inoculations of the PA-VRP were protected whereas 90% of the C57Bl/6 mice which received four inoculations were protected from an otherwise lethal challenge of *B. anthracis* (Sterne strain). The geometric mean of the serum ELISA titers to PA of C57Bl/6 mice given four doses of the PA-replicon was 150500, as compared to 264 for mice that were inoculated with the negative control replicon. Figure 3B shows ELISA titers and survival for A/J mice inoculated with two to four doses of PA-VRP. Three inoculations of the PA-VRP protected 70% of the A/J mice, whereas four inoculations protected 90% of the A/J mice from an otherwise lethal challenge of the *B. anthracis* (Sterne strain). The geometric mean of the serum ELISA titers against PA in A/J mice given four

doses of the PA-replicon was 223336 as compared to 100 for mice which were inoculated with the negative control replicon. As expected, none of the mice (from either strain) which were inoculated with the negative control replicon survived challenge with *B. anthracis* (Sterne strain).

Discussion/Conclusion

Since VEE virus replicates in the cytoplasm of eukaryotic cells, the VEE replicon vaccine vector is a useful tool for the expression of prokaryotic genes in eukaryotic cells. Cytoplasmic expression of genes alleviates the difficulties imposed by splicing and nuclear transport of mRNA. We used the VEE replicon as a way to express the prokaryotic PA gene in eukaryotic cells and to develop a new vaccine candidate against anthrax.

The human AVA contains mostly PA protein and is presumed to protect humans by eliciting an antibody response that can neutralize the PA portion of anthrax toxin produced by invading *B. anthracis*. After neutralization of the toxin, the immune system destroys the invading bacteria. In order to model the human disease, we chose to use mice and the Sterne strain of *B. anthracis*. The Sterne strain produces anthrax toxin (encoded on the pXO1 plasmid) but lacks a capsule (encoded on the pXO2 plasmid). Mice exposed to large quantities of Sterne (ranging from 10^3 to 10^8 spores for the different mouse strains) are not able to overcome the effects of the toxin produced by the bacteria so they succumb to infection. We hypothesized that by immunizing the mice with PA, thereby inducing neutralizing antibodies to the PA

portion of the toxin produced by a challenge inoculum, that the mice would be protected.

A VEE replicon was constructed which expressed the PA gene from *B. anthracis* and was evaluated for in vitro expression, in vivo immunogenicity, and protective efficacy. C57Bl/6 and A/J mice demonstrated increasing antibody responses to PA when inoculated with increasing doses of PA-VRP. The two mouse strains inoculated with up to four doses of PA-VRP displayed increased protection against a lethal challenge with *B. anthracis* (Sterne strain). The use of mice and the Sterne strain of *B. anthracis* useful as a model system for studying the immunogenicity and efficacy of replicon based anthrax vaccines.

15

20

25

30

EDITORIAL NOTE 55426/99

SEQUENCE LISTING PAGES 1/8 TO 8/8 ARE PART OF THE
DESCRIPTION AND ARE FOLLOWED BY CLAIM PAGES 22 TO 23.

SEQUENCE LISTING

<110> United States Army Medical Research Institute of
Infectious Diseases
Lee, John Scott
Pushko, Peter
Parker, Michael D.
Smith, Jonathan F.
Welkos, Susan L.

<120> Anthrax Vaccines

<130> 003/135/SAP

<140> US 09/350,729

<141> 1999-07-09

<150> US 60/092,416

<151> 1998-07-10

<160> 8

<170> Apple Macintosh Microsoft Word 6.0

<210> 1

<211> 2295

<212> DNA

<213> *Bacillus anthracis*

<220>

<400> 1

atgaaaaaac	gaaaagtgtt	aataccatta	atggcattgt	40
ctacgatatt	agtttcaagc	acaggtaatt	tagaggatgat	80
tcaggcagaa	gttaaacagg	agaaccgggtt	attaaatgaa	120
tcagaatcaa	gttcccaggg	gttactagga	tactatttta	160
gtgatttgaa	ttttcaagca	cccatgggtgg	ttacctcttc	200
tactacaggg	gatttatcta	ttcctagtct	tgagttagaa	240
aatattccat	cggaaaacca	atattttcaa	tctgctatct	280
ggtcaggatt	tatcaaagtt	aagaagagtg	atgaatatac	320
atttgctact	tccgctgata	atcatgtaac	aatgtgggta	360
gatgaccaag	aagtgattaa	ttaaagcttct	aattctaaca	400
aaatcagatt	agaaaaagga	agattatatc	aaataaaaat	440
tcaatatcaa	cgagaaaatc	ctactgaaaa	aggattggat	480
ttcaagttgt	actggaccga	ttctcaaaat	aaaaaagaag	520
tgatttctag	tgataactta	caattgccag	aattaaaaca	560
aaaatcttcg	aactcaagaa	aaaagacaag	tacaagtgct	600

SUBSTITUTE SHEET (RULE 26)

```
ggacctacgg ttccagaccg tgacaatgat ggaatccctg 640
attcattaga ggtagaagga tatacgggtg atgtcaaaaa 680
taaaagaact tttctttcac catggatttc taatattcat 720
gaaaagaaag gattaaccaa atataaatca tctcctgaaa 760
aatggagcac ggcttctgat ccgtacagtg atttcgaaaa 800
ggttacagga cggattgata agaattgtatc accagaggca 840
agacaccccc ttgtggcagc ttatccgatt gtacatgtag 880
aratggagaa tattattctc tcaaaaaatg aggatcaatc 920
cacacagaat actgatatgt aaacgagaac aataagttaa 960
aatacttcta caagtaggac acatactagt gaagtacatg 1000
gaaatgcaga agtgcattgc tcttctttg atattggtgg 1040
gagtgtatct gcaggattta gtaattcgaa ttcaagtacg 1080
gtcgaatttg atcattcact atctctagca ggggaaagaa 1120
cttgggctga aacaatgggt ttaaataccg ctgatacagc 1160
aagattaaat gccaatatta gatattgtaa tactgggacg 1200
gtctcaatct acaacgtgtt accaacgact tcgtagtggt 1240
taggaaaaaa tcaaacactc gcgacaatta aagctaagga 1280
aaaccaatta agtcaaatac ttgcacctaa taattattat 1320
ccttctaaaa acttggcgcc aatcgcatla aatgcacaag 1360
acgatttcag ttctactcca attacaatga attacaatca 1400
atctcttgag ttagaaaaaa cgaaacaatt aagattagat 1440
acggatcaag tatatgggaa tatagcaaca tacaattttg 1480
aaaaatggaag agtgagggtg gatacaggct cgaactggag 1520
tgaagtgtta ccgcaaattc aagaaacaac tgcacgtatc 1560
atctttaatg gaaaagattt aaatctggtg gaaaggcgga 1600
tagcggcggt taatcctagt gatccattag aaacgactaa 1640
accggatatg acattaaaag aagcccttaa aatagcattt 1680
ggatttaacg aaccgaatgg aaacttaca tatcaaggga 1720
aagacataac cgaatttgat tttaatttcg atcaacaaac 1760
atctcaaaat atcaagaatc agttagcgga attaaacgca 1800
actaacatat atactgtatt agataaaatc aaattaaatg 1840
caaaaatgaa tattttaata agagataaac gttttcatta 1880
tgatagaaat aacatagcag ttggggcgga tgagtcagta 1920
gttaaggagg ctcatagaga agtaattaat tcgtcaacag 1960
agggattatt gttaaattat gataaggata taagaaaaat 2000
attatcaggt tatattgtag aaattgaaga tactgaaggg 2040
cttaaagaag ttataaatga cagatatgat atgttgaata 2080
tttctagttt acggcaagat ggaaaaacat ttatagattt 2120
taaaaaatat aatgataaat taccgttata tataagtaat 2160
cccaattata aggtaaatgt atatgctgtt actaaagaaa 2200
acactattat taatcctagt gagaatgggg atactagtac 2240
caacgggatc aagaaaattt taatcttttc taaaaaaggc 2280
tatgagatag gataa 2295
```

<210> 2

<211> 3011

<212> DNA

<213> *Bacillus anthracis*

<220>

<400> 2

```
atggaagtta aacaggagaa ccggttatta aatgaatcag aatcaagttc
ccaggggtta ctaggatact attttagtga tttgaatttt caagcaccca
tgggtggttac ctcttctact acaggggatt tatctattcc tagttctgag
ttagaaaata ttccatcggg aaaccaatat tttcaatctg ctatttggtc
aggattttatc aaagttaaga agagtgatga atatacattt gctactttccg
ctgataatca tgtaacaatg tgggtagatg accaagaagt gattaataaaa
gcttctaatc ctaacaaaat cagattagaa aaaggaagat tatatcaaat
aaaaattcaa tatcaacgag aaaatcctac tgaaaaagga ttggatttca
agttgtactg gaccgattct caaaaataaaa aagaagtgat ttctagtgat
aacttacaat tgccagaatt aaaacaaaaa tcttcgaact caagaaaaaa
gcgaagtaca agtgcgtggac ctacgggtcc agaccgtgac aatgatggaa
tccctgattc attagaggta gaaggatata cggttgatgt caaaaataaa
agaacttttc ttccaccatg gatttctaatt attcatgaaa agaaaggatt
aaccaaatat aaatcatctc ctgaaaaatg gagcacggct tctgatccgt
acagtgattt cgaaaaaggt acaggacgga ttgataagaa tgatcacca
gaggcaagac acccccttgt gccagcttat ccgattgtac atgtagatat
ggagaatatt attctctcaa aaaatgagga tcaatccaca cagatactg
atagtgaac gagaacaata agtaaaaaa cttctacaag taggacacat
actagtgaag tacatggaaa tgcagaagtg catgcgtcgt tctttgatat
tgggtgggagt gtatctgcag gatttagtaa ttcgaattca agtacgggtc
caattgatca ttactatct ctacgagggg aaagaacttg ggctgaaaca
atgggtttaa ataccgctga tacagcaaga ttaaatgcc aatattagata
tgtaaaatact gggacggctc caatctacaa cgtgttacca acgacttcgt
tagtgttagg aaaaaatcaa acactcgcga caattaaagc taaggaaaaac
caattaaagc aaataacttg acctaataat tattatcctt ctaaaaactt
ggcgccaatc gcattaaatg cacaagacga tttcagttct actccaatta
caatgaatta caatcaattt cttgagttag aaaaaacgaa acaattaaga
ttagatacgg atcaagtata tgggaatata gcaacataca attttgaaaa
tggaaagagt aggggtggata caggctcgaa ctggagtga gtgttaccgc
aaattcaaga aacaactgca cgtatcattt ttaattggaa agatttaaat
ctggtagaaa ggcggatagc gccgggtta cctagtgtac cattagaaac
gactaaaccg gatatgacat taaaagaagc ccttaaaaata gcatttggat
ttaacgaacc gaatggaaac ttacaatatc aagggaaaga cataaccgaa
tttgatttta atttcgatca acaaactct caaaatatca agaatcagtt
agcgggaatta aacgcaacta acatatatac tgtattagat aaaaataaat
taaatgcaaa aatgaatatt ttaataagag ataaacgttt tcattatgat
agaaataaca tagcagttgg gccggatgag tcagtagtta agggaggctca
tagagaagta attaatcgt caacagaggg attattgtta aatattgata
aggatataag aaaaatatta tcaggttata ttgtagaaat tgaagatact
gaagggctta aagaagttat aaatgacaga tatgatatgt tgaatatttc
tagtttacgg caagatggaa aaacatttat agattttaaa aaatataatg
ataaattacc gttatatata agtaatccca attataaggt aaatgtatat
gctgttacta aagaaaacac tattattaat cctagtgaga atggggatag
tagtaccacac gggatcaaga aaattttaat cttttctaaa aaaggctatg
agataggata a
```

<210> 3

<211> 2292

<212> DNA

<213> *Bacillus anthracis*

<220>

<400> 3

```
atggatgcaa tgaagagagg gctctgctgt gtgctgctgc tgtgtggagc
agtcttcggt tcggctagcg aggtgattca ggcagaagtt aaacaggaga
accggttatt aaatgaatca gaatcaagtt cccaggggtt actaggatac
tatttttagtg atttgaattt tcaagcaccg atgggtggta cctcttctac
tacaggggat ttatctattc ctagtcttga gttagaaaat attccatcgg
aaaaccaata ttttcaatct gctatttggg caggatttat caaagttaag
aagagtgatg aatatacatt tgctacttcc gctgataatc atgtaacaat
gtgggtagat gaccaagaag tgattaataa agcttctaata tctaacaaaa
tcagattaga aaaaggaaga ttatatcaaa taaaaattca atatcaacga
gaaaatccta ctgaaaaagg attggatttc aagttgtact ggaccgattc
tcaaaataaa aaagaagtga tttctagtga taacttacia ttgcagaat
taaaacaaaa atcttcgaac tcaagaaaaa agcgaagtac aagtgtgga
cctacgggttc cagaccgtga caatgatgga atccctgatt cattagagggt
agaaggatat acggttgatg tcaaaaaata aagaactttt ctttcaccat
ggattttctaa tattcatgaa aagaaggat taaccaataa taaatcatct
cctgaaaaat ggagcacggc ttctgatccg tacagtgatt tcgaaaagggt
tacaggacgg attgataaga atgtatcacc agaggcaaga caccoccttg
tggcagctta tccgattgta catgtagata tggagaatat tattctctca
aaaaatgagg atcaatccac acagaatact gatagtgaac cgagaacaat
aagtaaaaat acttctacaa gtaggacaca tactagtga gtacatggaa
atcgagaagt gcatgcgtcg ttctttgata ttggtgggag tgtatctgca
ggatttagta attcgaattc aagtacggtc gcaattgatc attcactatc
tctagcaggg gaaagaactt gggctgaaac aatgggttta aataccgctg
atacagcaag attaaatgcc aatattagat atgtaaatac tgggacggct
ccaatctaca acgtgttacc aacgacttcg ttagtgttag gaaaaaatca
aacactcgcg acaattaaaag ctaaggaaaa ccaattaagt caaatacttg
cacctaataa ttattatcct tctaaaaact tggcgccaat cgcattaaat
gcacaagacg atttcagttc tactccaatt acaatgaatt acaatcaatt
tcttgagtta gaaaaaacga aacaattaaag attagatacg gatcaagtat
atgggaatat agcaacatac aattttgaaa atggaagagt gaggggtggat
acaggctcga actggagtga agtgttaccg caaattcaag aaacaactgc
acgtatcatt ttaattggaa aagatttaaa tctggtagaa aggcggatag
cggcgggttaa tctagtgat ccattagaaa cgactaaacc ggatatgaca
ttaaagaag cccttaaaat agcatttgga tttacgaac cgaatggaaa
cttacaatat caagggaaag acataaccga atttgatttt aatttcgatc
aacaacatc tcaaaatcag aagaatcagt tagcggaaat aaacgcaact
aacatatata ctgtattaga taaaatcaaa ttaaatgcaa aatgaatat
tttaataaga gataaacggt ttcatatga tagaaataac atagcagttg
gggaggatga gtcagtagtt aaggagggtc atagagaagt aattaattcg
tcaacagagg gattattggt aaatattgat aaggatataa gaaaaatatt
atcagggttat attgtagaaa ttgaagatac tgaagggtt aaagaagtta
taaataacag atatgatag ttgaatattt ctagtttacg gcaagatgga
aaaacattta tagattttta aaaaataaat gataaattac cgttatatat
aagtaatccc aattataagg taaatgtata tgctgttact aaagaaaaca
```

SUBSTITUTE SHEET (RULE 26)

ctattatttaa tcctagttag aatggggata ctagtaccac cgggatcaag
aaaatttttaa tcttttctaa aaaaggctat gagataggat aa

<210> 4

<211> 1710

<212> DNA

<213> *Bacillus anthracis*

<220>

<400> 4

atgagtacaa gtgctggacc tacgggtcca gaccgtgaca atgatggaat
ccctgattca ttagaggtag aaggatatac ggttgatgac aaaaaataaa
gaacttttct ttcaccatgg atttctaata ttcattgaaa gaaaggatta
accaaataa aatcatctcc tgaaaaatgg agcacggctt ctgatccgta
cagtgttttc gaaaagggtta caggacggat tgataagaat gtatcaccag
aggcaagaca ccccttcttg gcagcttacc cgattgtaca ttagatatg
gagaatatta ttctctcaaa aaatgaggat caatccacac agaatactga
tagtgaacg agaacaataa gtaaaaaaac ttctacaagt aggacacata
ctagtgaagt acatggaaat gcagaagtgc atgcgtcgtt ctttgatatt
ggtgggagtg tatctgcagg atttagtaat tcgaattcaa gtacggctgc
aattgatcat tcactatctc tagcagggga aagaacttgg gctgaaaaca
tggttttaaa taccgtgat acagcaagat taaatgcaa tattagatat
gtaaaactcg ggacggctcc aatctacaac gtgttaccaa cgacttcgtt
agtgttagga aaaaatcaaa cactcgcgac aattaaagct aaggaaaacc
aattaagtca aatacttgca cctaataatt attatccttc taaaaacttg
gcgccaatcg cattaaatgc acaagacgat ttcagttcta ctccaattac
aatgaattac aatcaatttc ttgagttaga aaaaacgaaa caattaagat
tagatacggg tcaagtatat gggaatatag caacatacaa ttttgaatat
ggaagagtga ggttggtatc aggtcgaac tggagtgaag tgtaccgca
aattcaagaa acaactgcac gtatcatttt taatggaaa gatttaaatc
tggtagaaag gcggtatgag gcggttaaac ctagtgatcc attagaaacg
actaaaccgg atatgacatt aaaagaagcc cttaaaatag catttgatt
taacgaaccg aatggaaact tacaatatca agggaaagac ataaccgaat
ttgattttta tttcgatcaa caaacatctc aaaatatcaa gaatcagtta
gcggaattaa acgcaactaa catatatact gtattagata aaatcaaat
aaatgcaaaa atgaatatat taataagaga taaacgtttt cattatgata
gaaataacat agcagttggg gcggtatgag cagtagttaa ggaggctcat
agagaagtta ttaattcgtc aacagaggga ttattgttaa atattgataa
ggatataaga aaaaatttat caggttatat ttagaataat gaagatactg
aagggtctaa agaagttata aatgacagat atgatatggt gaatatctt
agtttacggc aagatggaaa aacatttata gatttttaaa aatataatga
taaattaccg ttatatataa gtaatcccaa ttataaggta aatgtatatg
ctgttactaa agaaaacact attattaatc ctagtgagaa tggggatact
agtaccaacg ggatcaagaa aattttaatc ttttctaaaa aaggctatga
gataggataa

SUBSTITUTE SHEET (RULE 26)

<210> 5

<211> 763

<212> PRT

<213> *Bacillus anthracis*

<220>

<400> 5

1 MDAMKRGGLCCVLLLCGAVFVSASEVIQAEVKQENRLLNESESS
44 SQGLLGYYFSDLNFQAPMVVTSSTTGDLSSIPSELENIPSENQYFQSAIWSGFIKVKK
102
SDEYTFATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTKGLD
160
FKLYWTDSONKKEVISSDNLQLPELKQKSSNSRKKRSTSAGPTVPDRDNDGIPDSLEV
218
EGYTVDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNV
276
SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTSTSRHTTSEVHG
334
NAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNAN
392
IRYVNTGTAPIYVNLPTTSLVLGKNQTLATIKAKENQLSQILAPNNYPSKNLAPIAL
450
NAQDDFSSTPITMNYNQFLELEKTKQLRLDTDQVYGNIAITYNFENGRVVRVDTGSNWSE
508
VLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMTLKEALKIAFGFNEPN
566
GNLQYQKGDITEFDNFQQTSONIKNQLAELNATNIYTVLDKIKLNAKMNILIRDKR
624
FHYDRMNIAGADESVVKEAHREVINSSTEGLLLNIIDKDIRKILSGYIVEIEDTEGLK
682
EVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPYISNPYKVNKYAVTKENTIINPS
740 ENGDTSTNGIKKILIFSCKGYEIG

<210> 6

<211> 764

<212> PRT

<213> *Bacillus anthracis*

<220>

<400> 6

1 MKKRKVLIPLMALSTILVSSTGNLEVIQAEVKQENRLLNESESS

45
SQGLLGYFSDLNFQAPMVVTSSTTGDLSIPSELENIPSENQYFQSAIWSGFIKVKK
103
SDEYTFATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTKGLD
161
FKLYWTDSONKKEVISSDNLQLPELKQKSSNSRKKRSTSAGPTVPDRDNDGIPDSLEV
219
EGYTVDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKTGRIDKNV
277
SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTSTSRHTTSEVHG
335
NAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNAN
393
IRYVNTGTAPIYNVLPPTSLVLGKNQTLATIKAKENQLSQILAPNNYPSKNLAPIAL
451
NAQDDFSSTPITMNYNQFLELEKTKQLRLDQVYGNIAATYNFENGRVRVDTGSNWSE
509
VLPQIQETTARIIFNGKDLNLVERRIAAVNP SDPLETTKPDMTLKEALKIAFGFNEPN
567
GNLQYQGDITEFDNFDDQTSQNIKNQLAELNATNIYTVLDKIKLNAMNIIIRDKR
625
FHYDRNNAVGADES VVKEAHREVINSSTEGLLLNIIDKDIRKILSGYIVEIEDTEGLK
683
EVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPYISNPNYKVVYAVTKENTIINPS
741 ENGDTSTNGIKKILIFS KKGYEIG

<210> 7

<211> 736

<212> PRT

<213> *Bacillus anthracis*

<220>

<400> 7

1 MEVKQENRLLNESESS
17
SQGLLGYFSDLNFQAPMVVTSSTTGDLSIPSELENIPSENQYFQSAIWSGFIKVKK
75
SDEYTFATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTKGLD
133
FKLYWTDSONKKEVISSDNLQLPELKQKSSNSRKKRSTSAGPTVPDRDNDGIPDSLEV
191
EGYTVDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKTGRIDKNV
249
SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTSTSRHTTSEVHG
307
NAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNAN

SUBSTITUTE SHEET (RULE 26)

365
IRYVNTGTAPIYNVLPPTSLVLGKNQTLATIKAKENQLSQILAPNNYPSKNLAPIAL
423
NAQDDFSSTPITMNYNQFLELEKTKQLRLDQVYGNATYNFENGRVVRVDTGSNWSE
481
VLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMTLKEALKIAFGFNEPN
539
GNLQYQGDITEFDNFQDQTSQNIKNQLAELNATNIYTVLDKIKLNAKMNILIRDKR
597
FHYDRNNIAGADESVVKEAHREVINSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLK
655
EVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPYISNPYKVNVAVTKENTIINPS
713 ENGDTSTNGIKKILIFSKKGYEIG

<210> 8

<211> 569

<212> PRT

<213> *Bacillus anthracis*

<220>

<400> 8

1 MSTSAGPTVPDRDNDGIPDSLEV
24
EGYTVDVKNKRTFLSPWISNIHEKKGLTKYSSPEKWSTASDPYSDFEKTGRIDKNV
82
SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTSTSRHTTSEVHG
140
NAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNAN
198
IRYVNTGTAPIYNVLPPTSLVLGKNQTLATIKAKENQLSQILAPNNYPSKNLAPIAL
256
NAQDDFSSTPITMNYNQFLELEKTKQLRLDQVYGNATYNFENGRVVRVDTGSNWSE
314
VLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMTLKEALKIAFGFNEPN
372
GNLQYQGDITEFDNFQDQTSQNIKNQLAELNATNIYTVLDKIKLNAKMNILIRDKR
430
FHYDRNNIAGADESVVKEAHREVINSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLK
488
EVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPYISNPYKVNVAVTKENTIINPS
546 ENGDTSTNGIKKILIFSKKGYEIG

SUBSTITUTE SHEET (RULE 26)

What is claimed is:

1. A recombinant DNA construct comprising:
 - (i) a VEE virus replicon vector, and
 - (ii) at least one nucleic acid fragment comprising a nucleotide
 5 sequence encoding a *B. anthracis* protein selected from the group
 consisting of: PA, MAT-PA, TPA-PA, and PA63.
2. The recombinant DNA construct according to claim 1 wherein said VEE
 virus replicon vector is a eukaryotic vector.
3. The recombinant DNA construct according to claim 1 wherein said
 10 construct is p3014-PA.
4. The recombinant DNA construct according to claim 1 wherein said
 construct is p3014-TPA-PA.
5. The recombinant DNA construct according to claim 1 wherein said
 construct is p3014-MAT-PA.
6. The recombinant DNA construct according to claim 1 wherein said
 15 construct is p3014-PA63.
7. Self replicating RNA produced from a construct selected from the group
 consisting of: p3014-PA, p3014-TPA-PA, p3014-MAT-PA and p3014-
 P63.
8. Infectious alphavirus particles produced from packaging the self
 20 replicating RNA of claim 7.
9. A pharmaceutical composition comprising infectious alphavirus particles
 according to claim 8 in an effective immunogenic amount in a
 pharmaceutically acceptable carrier and/or adjuvant.

10. A host cell transformed with a recombinant DNA construct according to claim 1.
11. The host cell according to claim 10 wherein said host cell is prokaryotic.
- 5 12. The host cell according to claim 10 wherein said host cell eukaryotic.
13. A method for producing *B. anthracis* protein comprising culturing the cells according to claim 10 under conditions such that said DNA fragment is expressed and said protein is produced.
- 10 14. A method for producing *B. anthracis* protein comprising culturing the cells according to claim 12 under conditions such that said DNA fragment is expressed and said protein is produced.
15. A vaccine for anthrax comprising viral particles containing one or more replicon RNA encoding any combination of *B. anthracis* proteins selected from the group consisting of PA, PA-MAT, TPA-PA and PA63.
- 15 16. A pharmaceutical composition comprising the self replication RNA of claim 7 in an effective immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.
- 20 17. A pharmaceutical composition comprising one or more recombinant DNA constructs selected from the group consisting of: p3014-PA, p3014-TPA-PA, p3014-MAT-PA, and p3014-P63 in a pharmaceutically acceptable amount, in a pharmaceutically acceptable carrier and/or adjuvant.



FIG. 1

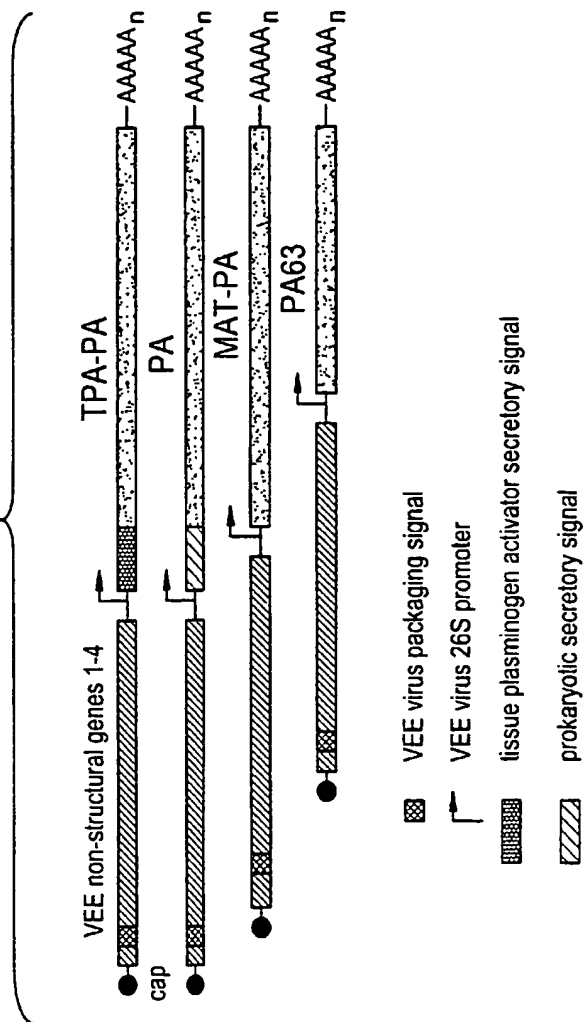


FIG. 2

lysate ^a	STD ^b	lysate ^c	lysate ^c	lysate ^c	Secretory Sequence
	-	+	+	+	

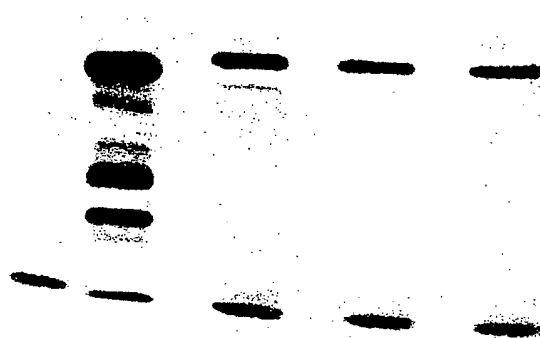


FIG. 3A

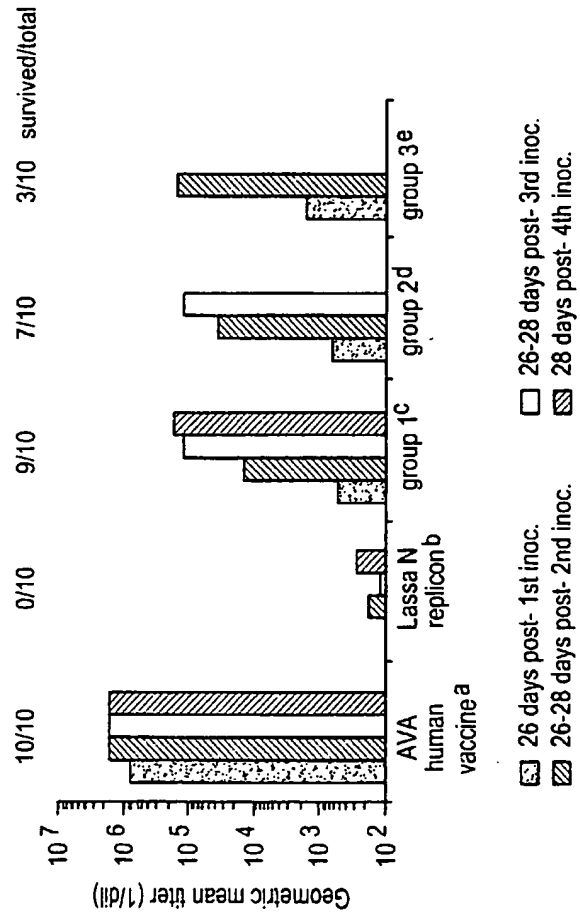


FIG. 3B

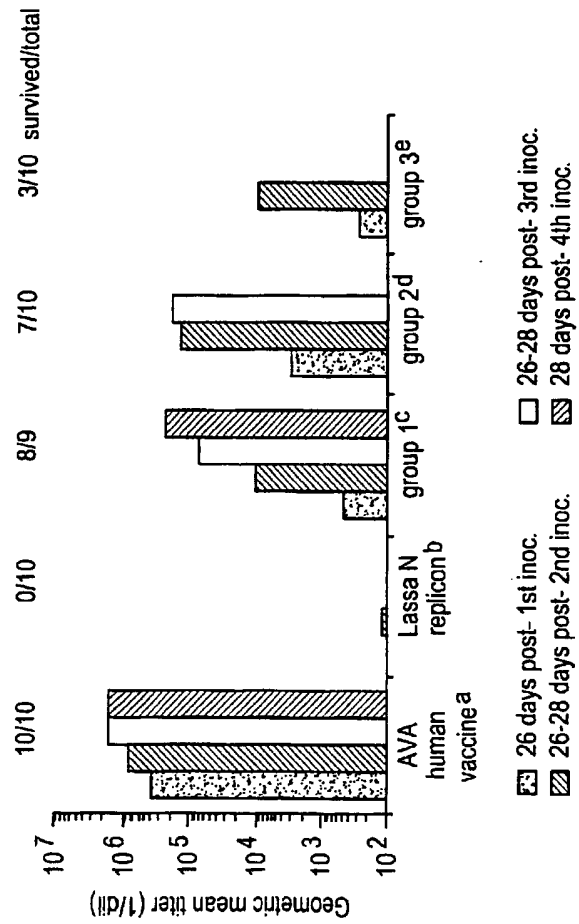
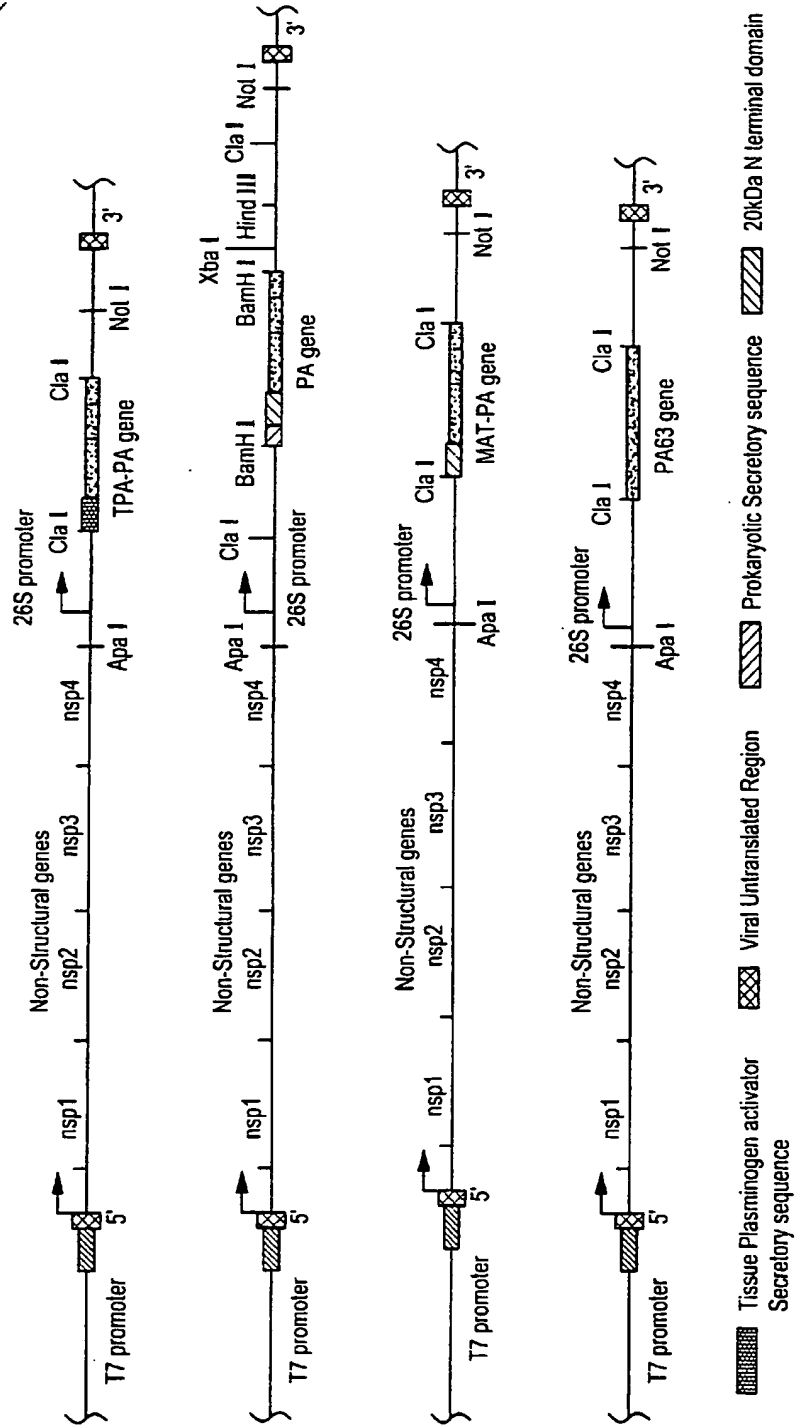


FIG. 4



THIS PAGE LEFT BLANK